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Published in:
Materials science & engineering c-Biomimetic and supramolecular systems

DOI:
10.1016/j.msec.2020.110930

Publication date:
2020

Materials Science & Engineering C 112 (2020) 110930

Contents lists available at ScienceDirect

Materials Science & Engineering C

journal homepage: www.elsevier.com/locate/msec

Effect of medium and aggregation on antibacterial activity of nanodiamonds

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ABSTRACT

Fluorescent nanodiamonds are widely used as abrasives, optical or magnetic labels, in drug delivery or nanoscale sensing. They are considered very biocompatible in mammalian cells. However, in bacteria the situation looks different and results are highly controversial. This article presents a short review of the published literature and a systematic experimental study of different strains, nanoparticle sizes and surface chemistries. Most notably, particle aggregation behaviour and bacterial clumping are taken into consideration to explain reduced colony counts, which can be wrongly interpreted as a bactericidal effect. The experiments show no mechanism can be linked to a specific material property, but prove that aggregation and bacteriostatic effect of nanodiamond attachment play a significant role in the reported results.

1. Introduction

Materials based on diamond nanoparticles are rapidly expanding their range of applications in biomedical research. These applications include their use as a versatile and biocompatible surface modification [1,2], carriers for drug delivery [3] and sensitive sensors for physical parameters such as temperature [4] and magnetic fields [5]. Owing to their carbon surface chemistry and inertness, nanodiamonds are suitable for the conjugation of a variety of biomolecules [6], while also exhibiting little to no toxicity in mammalian cells or larger organisms [7].

Interestingly, nanodiamond materials have also been investigated for their antibacterial properties. The rare combination of both biocompatibility and antibacterial activity would further increase the attractiveness of nanodiamond as a platform for developing biomaterials. However, successfully defending an environment from bacterial colonization and growth requires that many factors are taken into account. Clinical situations, food safety and water purification all impose different standards and limitations on how colonization of harmful microorganisms can be prevented or combatted. These strategies need to be tailored to the type and composition of the microbiota, the possible interactions with the host environment and the desired level of cleanliness or sterility among other factors.

The use of nanodiamonds as an antibacterial material has been investigated in different forms. However, the results are highly controversial. Most studies have made use of detonation nanodiamonds (DNDs), round particles of roughly 5 nm in diameter that are synthesized in the detonation of carbon rich explosives [8]. These have been used in pure form, as well as with various surface modifications [9]. The possibility of coating surfaces with diamond nanostructures has also been realized [1,2,10–12] and tested for the rate of bacterial colonization. Recently, our group investigated the interaction of bacteria with milled diamond particles in various media [13]. These particles with a larger variation in size and geometry compared to DNDs, can contain fluorescent color centers for biosensing. Their surface is less reactive and thus they are used for different existing industrial applications. Our results showed that antibacterial effects were evident in *Staphylococcus aureus*, even though that results of short experiments could not be readily extrapolated to longer exposure, different bacterial strains or different media.

The many forms in which diamond nanoparticles can be used and integrated into biomaterials invite a more in-depth study of their interaction with bacteria. Several publications have reported an antibacterial effect of some extent that was attributed to the properties of (nano)diamond [14–18]. Their interaction with bacteria has been linked to chemical surface groups on the diamond, including acid anhydride [19] and carboxylic acid [20], but also possible internalization and mechanical disruption of the cell wall. Since bacterial attachment, growth and viability are dependent on many factors; it is not surprising that the observed results could not be ascribed to a single working mechanism. However, improving the understanding of the interplay between diamond materials, bacterial growth and the environment is quite valuable to the further development of diamond-based bio-applications.

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https://doi.org/10.1016/j.msec.2020.110930
Received 24 July 2019; Received in revised form 6 March 2020; Accepted 2 April 2020
Available online 03 April 2020

For this understanding, it is of great importance to understand what outcome is desired by the potential application of the material. Detonation nanodiamonds, milled nanodiamonds and diamond-modified surfaces are respectively researched for (among others) drug delivery, biosensing and implant coating. While some of these applications may benefit from bactericidal activity, inertness or attachment prevention are more important for others. Along with a large variety of materials and bacterial strains, there are thus also many methods that can be used to assess the interaction between them. Table 1 in the Supplementary information S.01 provides an overview of studies on the interaction of non-functionalized (nano)diamond materials with bacteria, summarizing their methods and respective outcomes.

The existing literature suggests that aggregation of nanodiamonds and their attachment to bacterial cell walls are key factors to their growth inhibiting the effect. The surface chemistry of the nanodiamonds, as well as the electrolyte concentration and composition, determine to which extent an antibacterial effect is observed. In this paper, we investigate in more detail the role of the cell wall type and medium composition in the interaction of milled nanodiamonds with Gram-positive and Gram-negative bacterial strains.

2. Experimental section

2.1. Material and methods

2.1.1. Nanodiamond materials

Monocrystalline milled HPHT nanodiamonds were obtained from Microdiamant AG (Lengwil, Switzerland) in four different nanoparticle sizes (median hydrodynamic diameter 125, 75, 25 and 18 nm). Since the last step in the production is cleaning in oxidizing acid, the surface is oxygen terminated. These particles typically have a negative zeta potential. For comparison of nanodiamond particles with different surface chemistry, detonation nanodiamonds (DND), carboxylated DND (cDND), fluorescent nanodiamonds (FNDs) and carboxylated FNDs (cFNDs) were purchased from Adamás Nanotechnologies (NC, USA).

For biological experiments, four different mass concentrations of all of NDs suspensions (500, 100, 10 and 1 μg mL^{-1}) were prepared by making serial dilutions from stock suspensions. Then the suspensions were sterilized (in the autoclave at 121 °C for 15 min) and tested for their antibacterial potential. The particle size distribution and hydrodynamic diameter of NDs suspensions were determined using a Malvern ZetaSizer Nano system (Malvern Instruments Ltd., Malvern, UK, www.malvern.com). X-ray photoelectron spectroscopy (XPS) measurements were performed to characterization of surface chemistry.

2.1.2. Chemicals and media

All growth media were purchased from Oxoid (Basingstoke, United Kingdom), while Bacto agar and Bacto peptone were purchased from BD (Le Pont de Claix, France). Foetal bovine serum (FBS) and other chemicals were purchased from Gibco Life Technologies (Bleiswijk, the Netherlands) and Sigma (St. Louis, MO) or Merck (Darmstadt, Germany), respectively.

2.1.3. Bacteria strain and culture conditions

Staphylococcus and *Escherichia* are among the most widespread genera of Gram-positive and Gram-negative bacteria, respectively, that induce food poisons and clinical infections. Bacterial strains used in this study including *Escherichia coli* ATCC 8739, *Staphylococcus aureus* ATCC 12600 and *Staphylococcus epidermidis* ATCC 12228 selected which are a Gram-negative bacterium, a Gram-positive bacterium with high and low Extracellular Polymeric Substance (EPS) excretion ability, respectively. All strains were stored at −80 °C in 7% DMSO. Precultures of strains were made by adding one grown colony on Sheep Blood Agar plates to 10 mL of growth medium followed by incubation in a shaking incubator (150 rpm) at 37 °C for 24 h. Main cultures were prepared by taking 2 mL of the preculture into 40 mL sterile growth medium and incubating for 16 h. The main cultures were then washed twice with phosphate buffered saline, intermitted by centrifuging at 6500 *g* and followed by sonication to break apart bacterial aggregates. The cells were then enumerated using a Bürker-Türk counting chamber.

2.1.4. Dynamic light scattering (DLS)

Dynamic light scattering (DLS) is a common technique for determining particle size distribution and hydrodynamic diameter in colloidal suspensions. In this study, characterization of size and aggregation formation of nanoparticles was performed through DLS measurements using a Malvern ZetaSizer Nano system (Malvern Instruments Ltd., Malvern, UK). The ZetaSizer measures the size of suspended colloidal particles by determining their Brownian motion using DLS.

2.1.5. Minimum inhibitory concentration (MIC) assay

The minimum inhibitory concentration (MIC) is the most common method used to determine antimicrobial activity. The MIC is defined as the lowest concentration of a test suspension where no growth is visible with the naked eye. In this work, the MIC protocol was carried out in 96-well plates for three bacteria strains (*E. coli*, *S. aureus* and *S. epidermidis* high and low EPS secretion ability, respectively), involved pipetting 100 μl of each sterile test suspensions of NDs at different concentrations ranging from 2 to 10,000 μg mL^{-1} into wells. Inoculation was performed with 100 μl of bacteria suspensions (2×10^5 mL^{-1}) in the wells. Wells with growth medium but no NDs are referred as negative controls. The plates were incubated in a shake incubator (150 rpm) for 24 h at 37 °C. After the incubation, MIC was taken as the lowest concentration that inhibits bacterial growth completely. All of the experiments were performed in duplicates with each different bacterial strain on two different days.

2.1.6. Bacterial colony forming ability (CFA) assay

The primary step in order to use novel nanomaterials for *in vivo* investigations is the evaluation of their biocompatibility with cells and bio-surrounding. Consequently, in the case of bacteria, we started an investigation by measuring Colony Forming Ability in this study. To assess the CFA, a concentration of 2×10^8 ml^{-1} of all bacteria strains were individually exposed to several types of NDs suspensions (milled NDs, DND, cDND, FNDs and cFNDs). We also tested the effect in different media (full bacteria medium and full bacterial medium + 10% FBS). All samples were incubated in the respective media (Tryptone Soy Broth and Luria-Bertani broth were used for *Staphylococcus* and E*scherichia*, respectively) at 37 °C. Bacteria in media without NDs served as control experiments. After incubation for 60 min, the suspensions were diluted in a serial dilution down to a factor 10^{-5}, plated on plates containing agar respective medium and incubated at 37 °C. The number of colony forming units (CFU) was then counted the next day [11].

The interaction of differently sized and concentrated milled NDs with *S. aureus* and *S. epidermidis* was investigated. For this purpose, four different ND sizes (125 nm, 75 nm, 25 nm and 18 nm) in four different mass concentrations (500, 100, 10 and 1 μg mL^{-1}) were used. In addition, to see the effect of different media components on these strains, two combinations were tested: 16 ND/full medium and 16 ND/full medium + 10% FBS. The same experiment was performed with DND and cDND/full medium and FND and cFND/full medium suspensions using four different mass concentrations (500, 100, 10 and 1 μg mL^{-1}) to investigate the antibacterial effects of different types of NDs on *S. aureus* cells in medium.

2.1.7. SEM imaging

Scanning Electron Microscopy (SEM) was performed to achieve more details on the interaction of NDs and two bacterial strains with different types of cell wall. Sample preparation was done as described by Ong et al. [13]. Prior to SEM imaging, sample stages were sputtered with 30 nm gold layer and a Philips XL30 instrument was used to image at 10 kV.

2.1.8. Statistical analysis

All data points were expressed as mean values ± standard deviations with $n = 2$ or $n = 3$. Statistical analysis was performed using Origin 8.0 software by one-way ANOVA followed by Tukey's test. Statistical significance was considered at a value of $p < .05$.

3. Results and discussion

3.1. Role of particle aggregation and attachment on bacterial viability

Here, our aim was to investigate the influence of proteins and salt in the bacterial growth medium on the aggregation of NDs. The size of NDs in suspension with different compounds of the growth medium was measured by Dynamic light scattering (DLS). Medium compounds included Bacto™ Peptone (BP), NaCl, K_2HPO_4 and glucose. Bacto™ Peptone consists of pancreatic digest of casein and enzymatic digest of soybean and is used as an organic nitrogen source in microbiological culture media for cultivation of bacteria and fungi. A previous study on the interaction between DMEM medium and NDs by Hemelaar et al. [21] found that adding FBS to the medium at first and then adding the medium is effectively reducing the aggregation. Therefore, we investigated complemented medium with FBS (10%). Additionally, we tested NDs in Deionized water (A) as a reference; the other media were (B) Growth medium (BP + NaCl + K_2HPO_4 + Gls), (C) Growth medium + 10% FBS, (D) 100% BP, (E) BP + 10% FBS, (F) NaCl + K_2HPO_4 + Gls, (G) NaCl + K_2HPO_4 + Gls + 10% FBS. We prepared the samples by dispersing the NDs (125, 75, 25 and 18 nm) at a concentration of 100 μg mL^{-1} in all media, then their size distribution was analysed by DLS at room temperature. Fig. 1 shows the average hydrodynamic diameters of nanodiamond aggregates resulting from suspension in the presence of different components of media. The raw DLS data is presented in the Supplementary information S.02.

As expected, NDs in DI-water have the lowest hydrodynamic diameter. Therefore, we conclude that the aggregation of NDs doesn't occur in water. By adding the different components, ND aggregate diameters increased due to the presence of salts and proteins. It is well known that the presence of NaCl in solution results in strong and rapid

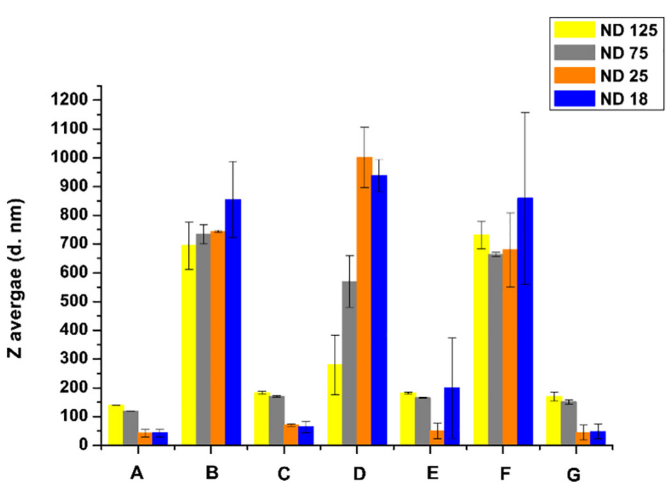

Fig. 1. Average hydrodynamic diameters of milled ND aggregates (18, 25, 75 and 125 nm) at the concentration of 100 μg mL^{-1} in different suspension media. A: DI-water, B: Growth medium (Bacto Peptone + NaCl + K_2HPO_4 + Gls), C: Growth medium + 10% FBS, D: 100% BP, E: BP + 10% FBS, F: NaCl + K_2HPO_4 + Gls, G: NaCl + K_2HPO_4 + Gls + 10% FBS. (Each column for all sizes is the mean of samples from two replicate experiments).

aggregation. The addition of 10% foetal bovine serum (FBS), containing a large variety of solutes including glucose, albumin, urea, cholesterol and salts effectively prevented and reverted aggregation. This suggests the presence of compounds in FBS with a strong tendency to coat the diamond surface and preventing aggregate formation. In the medium we used for further experiments, both protein compounds (Bacto Peptone), as well as the combination of salts and glucose, contributed to the aggregation of the nanodiamonds.

CFA experiments were performed on three different bacterial strains, *S. aureus* ATCC 12600, *S. epidermidis* ATCC 12228 and *E. coli* ATCC 8739. The former was identified as a high-level EPS producing strain, leading to strong and easy attachment to surfaces and presumably nanoparticles. *S. epidermidis* ATCC 12228 is a similar microbe (Gram positive *Staphylococcus*), but produces low levels of EPS. *E. coli* ATCC 8739 is a well-studied strain known to show little to no clumping or affinity with surfaces. Bacterial viability in the presence of different sizes and concentrations of milled NDs in the growth medium were assessed after 60 min incubation at 37 °C.

As it is revealed in Fig. 2A and C, addition of nanodiamonds results in dose-dependent inhibition of colony forming ability of both *S. aureus* and *S. epidermidis*. In other words, while colony forming ability of bacteria was independent of the size of NDs, by increasing the amount of NDs followed by one-hour incubation, the ability of bacteria to form colonies was decreased. Furthermore, as it is shown in Fig. 2C and D, addition of FBS to the growth medium negates the antibacterial effect of nanodiamonds. Although other effects resulting from the addition of FBS cannot be ruled out, this experiment shows a correlation between the nanodiamond's aggregation state and reduction in viability. The most notable difference between *S. aureus* and *S. epidermidis* is that a stronger reduction in colony count is already observed in *S. epidermidis* for lower (10 μg mL^{-1}) ND concentrations. In both cases, the addition of FBS changed the interaction of nanodiamond particles with bacteria. In combination with the DLS data this suggests a strong affinity of the nanodiamonds with some of the components in FBS, which is supported by previous experiments by Hemelaar et al. [21] However, one cannot make the distinction between contact killing by chemical surface groups and a different mode of interaction that is also dependent on the surface properties of the nanodiamond with this observation.

Fig. 3 shows the results of CFA measurements using *E. coli* ATCC 8739, four different ND sizes in four concentrations in growth medium (LB). There are no significant differences between larger and smaller ND particles in all concentrations (1–500 μg mL^{-1}). In addition, no significant differences were found for NDs at any size when increasing the ND concentrations from 1 to 500 μg mL^{-1}. This is consistent with the expectation that affinity for attachment of *E. coli* to surfaces and nanoparticles, as well as their tendency for clumping is low. These observations are supported by earlier work carried out by Ong et al. [13], where the CFA was assessed in phosphate buffered saline (PBS) for *S. aureus* and *E. coli*. This is attributed in part to the fact that Gram-negative strains exhibit low affinity with the nanodiamonds of any size, at any concentration and in any type of media.

3.2. Nanodiamonds show no MIC for Gram-positive and Gram negative bacteria

In order to determine true antibacterial or bactericidal activity, three different bacterial strains were exposed to increasing concentrations of nanodiamonds. After incubation for 24 h, bacterial growth was assessed by observation of turbidity. These experiments showed that despite the sharp reductions in colony forming ability after only 1 h of incubation in CFA experiments, there was no complete inhibition of bacterial growth for concentrations < 1000 mg/mL. This observation raises the question of what kind of interaction may lead to such a rapid reduction in CFAs on the short term, while showing no effects at longer times. In the next sections of this paper, we will explore several hypotheses for this question, which include proposed mechanisms based

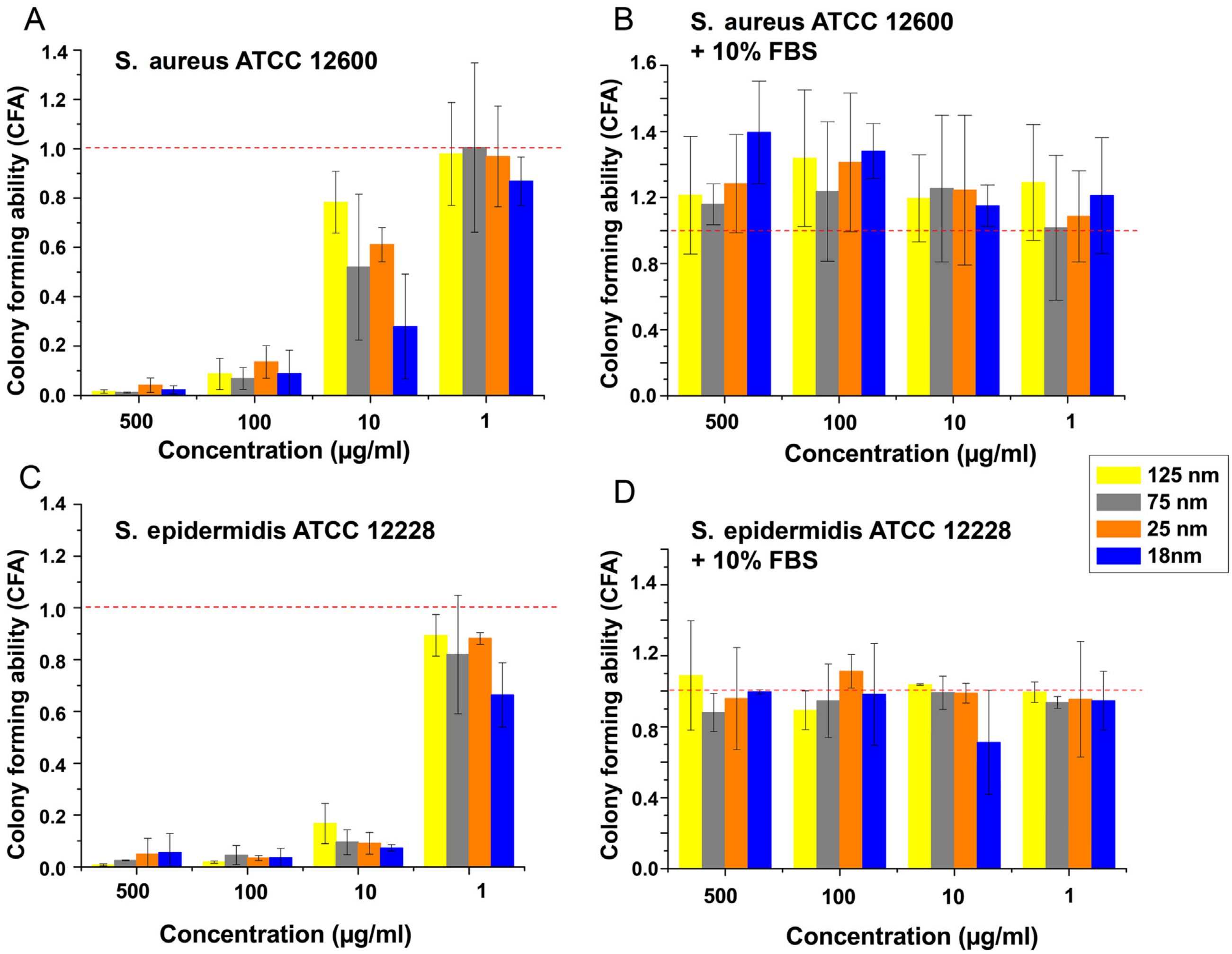

Fig. 2. *S. aureus* and *S. epidermidis* Colony forming ability in the presence of different concentrations and different sizes of NDs in full medium and full medium + 10% FBS. Colony forming ability of *S. aureus* in the presence of 1–500 µg mL^{-1} of NDs with different size (125, 75, 25 and 18 nm) in A) full medium and B) full medium + 10% FBS. Colony forming ability of *S. epidermidis* in the presence of 1–500 µg mL^{-1} of NDs with different size (125, 75, 25 and 18 nm) in C) full medium and D) full medium + 10% FBS. (CFA of control experiment = 1 for each condition. Each column in A and B graphs is the mean of samples from three replicate experiments).

on nanoparticle surface chemistry and aggregation behaviour.

3.3. Microscopic interaction of bacteria strains with NDs

The interaction of the examined bacteria with NDs was imaged using SEM. Fig. 4 shows a selection of SEM images with different magnifications obtained using *S. aureus* ATCC 12600 and *S. epidermidis* ATCC 1228 (high and low EPS producer, respectively) exposed 10 µg/mL 125 and 18 nm NDs. Bacteria suspensions without NDs were used as negative control. They illustrate no mechanical damages at interaction between bacteria and NDs. Apparently, there is no difference in interaction of NDs with both strains. SEM images of *S. aureus* cells with high EPS producing ability also show that the ND/bacteria interaction is more than ND/ND compared to *S. epidermidis*.

3.4. Surface chemistry

The surface chemistry of acid treated and non-acid treated fluorescent nanodiamonds (FNDs, Adamás Nanotechnologies, 40 nm hydrodynamic diameters) was characterized by X-ray Photoelectron Spectroscopy (XPS). These nanodiamonds are also of the milled type and are fluorescent due to a higher concentration of NV^- defects in the bulk, without further consequences for their relevant chemical and physical properties. Sample preparations were done by deposition on a glass coverslip and desiccation of the aqueous suspensions as provided by the manufacturer. The wide scan and high-resolution spectra of the C-1s and O-1s peaks are shown in Fig. 5.

The presence of acidic surface groups on nanodiamond, including acid anhydride and carboxylic acid, has been suggested as the origin of antibacterial activity against both Gram positive [19] and Gram negative [19,20] bacteria. Here, we have compared the XPS analysis of 40 nm FNDs that underwent treatment with nitric and sulfuric acids to increase the amount of carboxylic acid groups (from here: cFNDs) and untreated 40 nm nanodiamonds. Elemental analysis shows an oxygen-to-carbon ratio on the untreated nanodiamonds of 12.5%, compared to 61.3% for the cFNDs. The carboxylated sample contained significant content of Si (11.5%), N (3.5%) and Na (2.2%), along with traces amount of K, Ti and Zn. A high resolution scan of the C1s peaks revealed a 4% increase in groups at binding energies associated with C—O and O—C═O bonds. A shift to higher binding energies is also observed. In order to explore the role of the surface chemistry on interaction between nanodiamonds and bacteria, the assessment of colony forming ability of *S. aureus* ATCC 12600 was performed with FNDs, carboxylated FND (cFND), DND and carboxylated DND (cDND) at different concentrations. In several studies, the surface chemistry of DNDs has been linked to a notable antibacterial effect of different Gram-negative

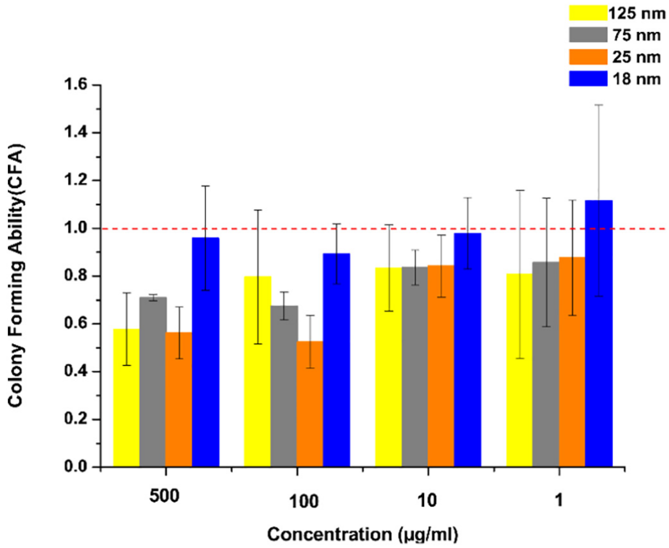

Fig. 3. Comparison of *E. coli* Colony forming ability in the presence of different concentrations and different sizes of NDs in growth medium. Colony forming ability of *E. coli* in the presence of 1–500 µg ml^{-1} of NDs with different size (125, 75, 25 and 18 nm) in medium. (CFA of control experiment = 1 for each condition. Each column in the graph is the mean of samples from two replicate experiments).

and Gram-positive strains. We assessed the effect of milled fluorescent NDs (FNDs), DNDs and carboxylated DNDs and FNDs (cDND, cFND) on *S. aureus* ATCC 12600.

Fig. 6 shows the CFA of *S. aureus* ATCC 12600 incubated with these types of NDs in a triplicate experiment. It can be seen that the difference between carboxylated and uncarboxylated surface is more pronounced in DNDs that have a much higher surface to volume ratio. This difference however is only prominent at the 10 µg mL^{-1} concentration. Interestingly, the difference in antibacterial activity between the two surface types fades at higher concentrations and even seems to slightly (not significantly) reverse at the highest concentration of 500 µg mL^{-1}. It is therefore unlikely that there is a direct killing effect of the carboxylated diamond surface, since one would expect to observe a steadier decrease of viability with increasing concentration. This observed effect may still be attributed to the altered surface chemistry affecting the particle's affinity for attachment to the bacterial cell wall, as has been suggested by other studies [12,13].

3.5. Deaggregation by sonication treatment

As a next step, we tried to investigate in more detail the role of environmental factors on the occurrence of NDs/bacteria aggregation and its influence on colony forming ability of bacteria. Therefore, the same experiment was performed but with the interference of sonication treatment and using a tube instead of microplates. As before, the results were compared with untreated samples. For this purpose, *S. aureus* ATCC 12600 with the initial concentration of 2×10^8 mL^{-1} was exposed to NDs suspensions (125, 75, 25 and 18 nm milled NDs) in full medium. The final concentration of NDs was 100 µg mL^{-1}. Bacteria in medium without NDs served as control experiments. After incubation at 37 °C for 60 min, these samples were divided into two different groups. One group was exposed to sonication (3 ∗ 10 s). For the second group, the sonication step was skipped. The suspensions were serially then diluted, plated on TSA plates and incubated at 37 °C. Subsequently, CFU was counted after one day.

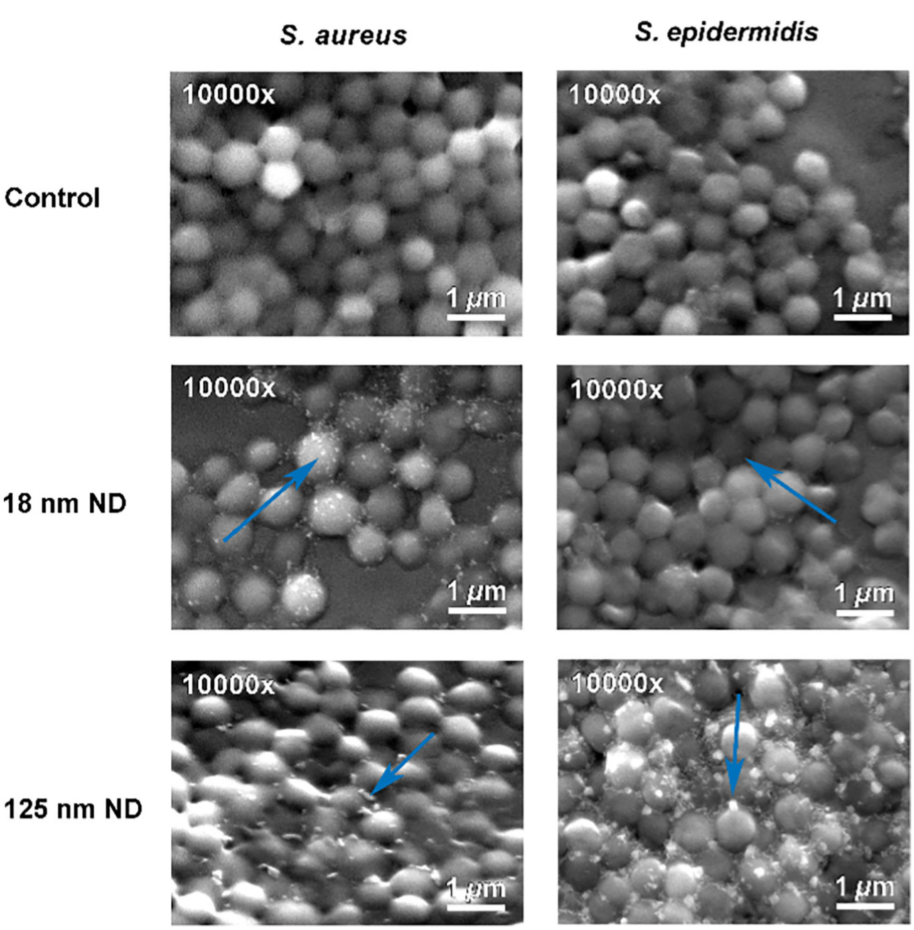

Fig. 4. Selection of SEM images at different magnifications show the interaction of bacteria strains (A) *S. aureus* with thick EPS and (B) *S. epidermidis* with thin EPS layer with nanodiamonds. Bacterial suspensions without NDs served as negative control. Milled NDs (18 nm and 125 nm) at 10 µg mL^{-1} were used for incubation prior to electron microscopy. Blue arrows indicate several locations where nanodiamond aggregates are attached to the bacterial cell wall. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

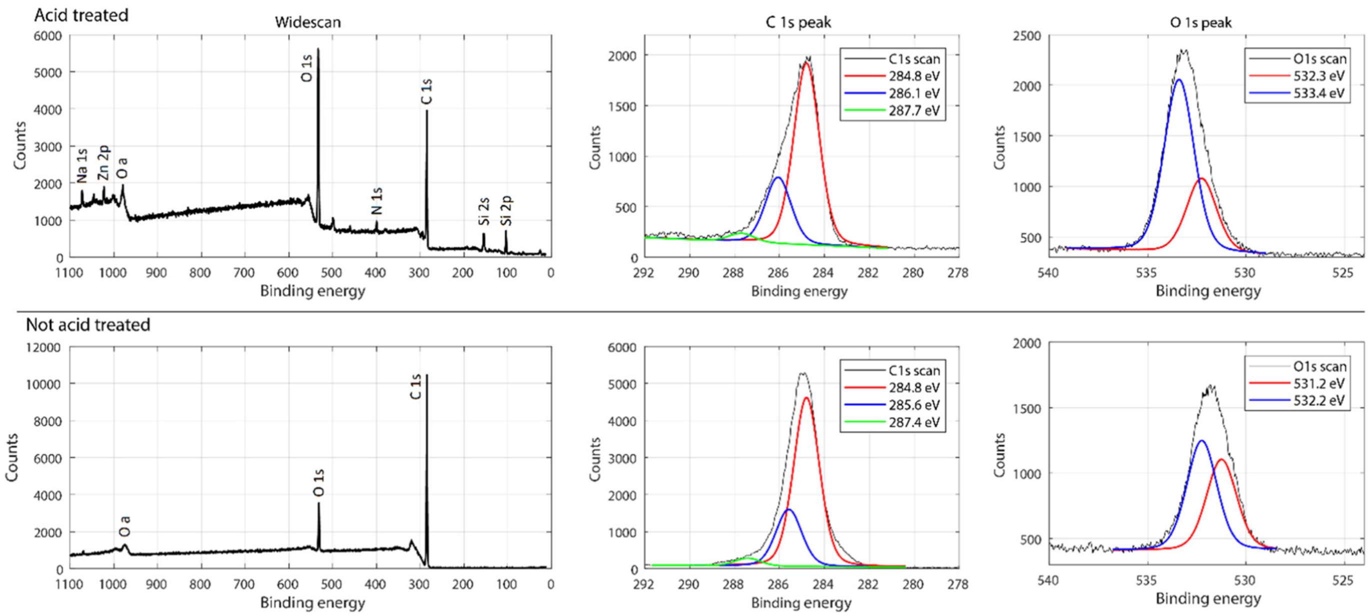

Fig. 5. XPS analysis of 40 nm fluorescent nanodiamonds treated with nitric and sulfuric acids (cFND, upper) *versus* non-acid treated 40 nm fluorescent nanodiamonds (FND).

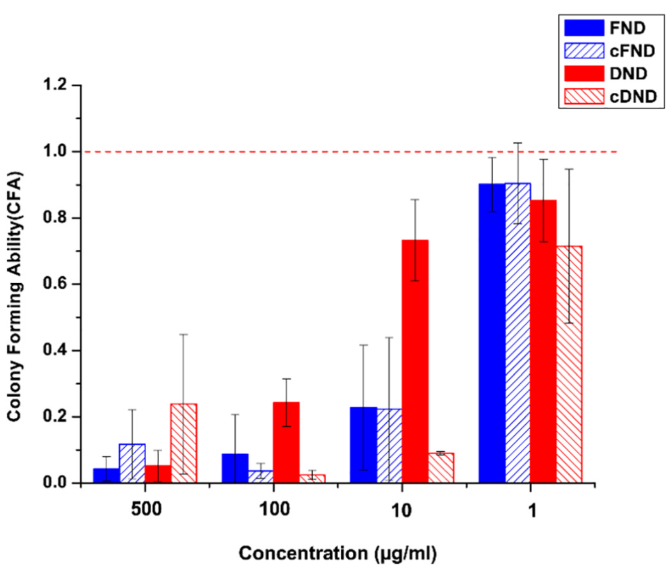

Fig. 6. Colony forming ability (CFA) of *S. aureus* ATCC 12600 after exposure to four types of nanodiamond (FND, cFND, DND and cDND) with different surface chemistry. The graph displays values relative to the control experiment for all tested concentrations (1–500 μg mL^{-1}). (CFA of control experiment = 1 for each condition. Each column in the graph is the mean of samples from three replicate experiments).

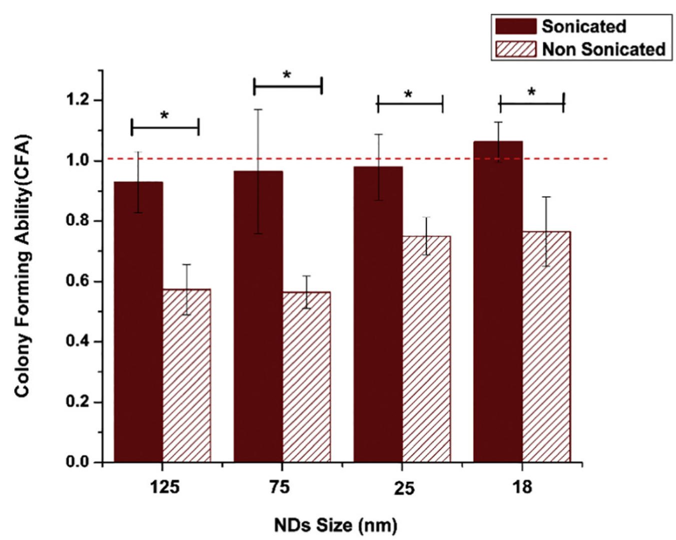

Fig. 7. Colony forming ability of *S. aureus* ATCC 12600 in presence of 100 μg mL^{-1} of NDs with different size (125, 75, 25 and 18 nm) in growth medium with and without de-aggregation by sonication before plating on agar. This experiment was done in triplicate. (*$p \leq .05$).

Data in Fig. 7 represent the average CFA of *S. aureus* ATCC 12600 in triplicate. The figure shows that the average CFA of *S. aureus* in sonicated samples is equal to that of the control for all sizes of milled NDs. However, the colony forming ability of *S. aureus* in non-sonicated samples is significantly ($p < .05$) smaller compared to sonicated one. The observed results obviously show the role of NDs/bacteria aggregation on CFA of Gram-positive bacteria, although the addition of FBS likely prevents aggregation regardless of what else is in the suspension.

4. Conclusions

Determining not only what the effect is of nanomaterials on bacterial growth, but also how this interaction takes place is essential for applicability. In this paper, we identify aggregation as an important factor in reducing the *in vitro* colony forming ability of *S. aureus* when exposed to milled nanodiamonds.

In earlier work, it was shown that colony forming ability of *S. aureus* ATCC 12600 was sharply reduced when exposed to concentrations of 10–100 mg/mL of milled nanodiamonds in phosphate buffered saline [13]. However, observations made in experiments with higher ND concentrations, or in DI water as suspension medium did not yield results consistent with a 'contact killing' model. Furthermore, metabolic rates were not reduced under conditions that significantly reduced the colony count. Therefore, killing on contact is deemed an unlikely mechanism. Fig. 8 shows the data from our previous work.

Based on the experiments carried out to scientific evidence for mechanism presented in this paper, we propose a model to explain the reduction in colony forming units based on aggregation of bacteria and

	Milled ND				Milled ND (Carboxylated)				Milled ND + FBS				DND				DND (Carboxylated)			
Conc. (µg/ml)	500	100	10	1	500	100	10	1	500	100	10	1	500	100	10	1	500	100	10	1
S. aureus	--	--	-	+	--	--	--	+	+	+	+	+	--	-	+/-	+	-	--	-	+/-
S. epidermidis	--	--	--	+	-	--	-	+	+	+	+	+								
E. coli	+/-	+/-	+	+																
S. aureus [a]	+/-	--	+/-	+																
E. coli [a]	+	+	+	+																
S. aureus [a]	+	++	++	+																
E. coli [a]	+	+	+	+																

++ Increased CFA
+ No decrease in CFA
+/- ≤ 50% decrease
- ≤ 90% decrease
-- ≥ 90% decrease

Culture medium
Phosphate buffered Saline
De-ionized water

Fig. 8. Summary of qualitative colony count data of the experiments performed in this study and ([a]) obtained in an earlier study by Ong et al.

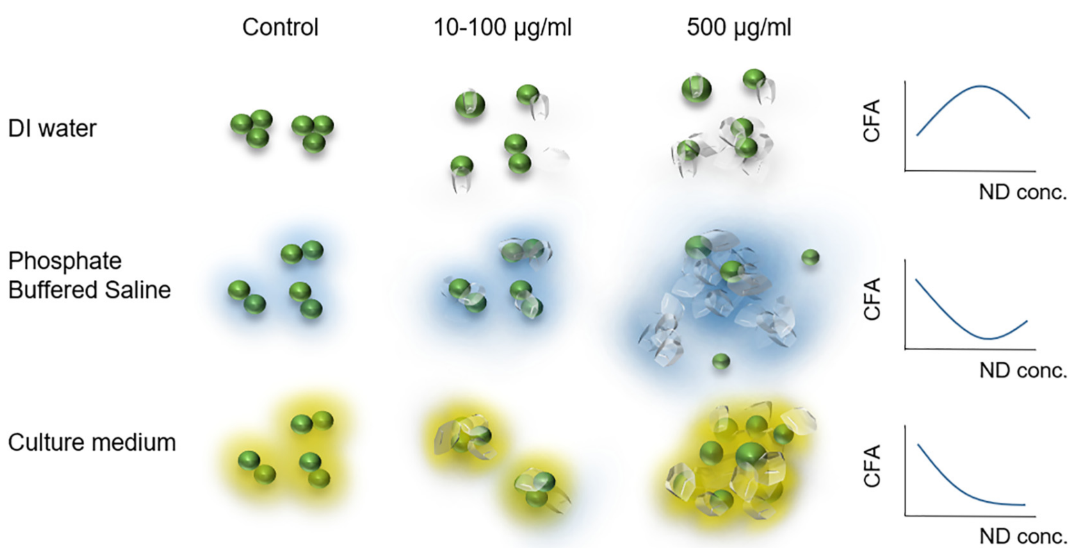

Fig. 9. Graphic model of aggregation processes occurring in different suspension media and their effect on the colony forming ability of *S. aureus*.

nanodiamonds in different suspension media (Fig. 9). In this model, the effect of the nanodiamonds is twofold. When nanodiamonds attach to the bacterial cell wall in a sufficient concentration, they inhibit proliferation into colonies. In addition, since nanodiamonds facilitate the formation of larger clusters of bacteria and nanodiamonds, result in reduction in the total amount of loose bacteria that can form separate colonies in an experiment.

In this model, nanodiamond concentration, size, suspension solutes and bacterial strain all play a role in determining the outcome of a colony counting experiment. Differences in colloidal stability of *S. aureus* ATCC 12600 can already be seen without addition of NDs, as the cells sediment more rapidly in DI water compared to PBS buffer. In contrast, nanodiamonds are stable in DI water suspensions but aggregate immediately in NaCl containing solutions. This difference is used to explain the outcome of previous work, where the addition of NDs triggers an opposite effect in the colony forming ability.

The experiments of this work were performed in a protein rich medium, where a large variety of compounds is present to facilitate aggregation of both bacteria and NDs. As opposed to PBS-buffer, which mainly aggregates NDs, colony forming ability in full medium is reduced further at ND concentrations of 500 µg mL^{-1}. Furthermore, we show that de-aggregation negates the reduction in colony forming ability. Although the addition of FBS likely leads to a shielding of the ND's chemical surface groups and therefore introduces additional factors other than de-aggregation, sonication clearly shows that NDs contribute significantly to clumping of the bacteria.

Rather than outlining the potential use of the observed effects, the conclusion of this work emphasizes the complexity of assessing the effect of new nanomaterials on bacteria. In our work, we identified aggregation as a multifactorial process that can easily lead to misinterpretation of outcomes. Without a basal understanding of how nanoparticles may interact with bacteria, claiming antibacterial activity requires broad evidence involving various strains and experimental conditions. We have shown here that contact killing by NDs is most likely not the interaction that leads to reduced colony counts in *S. aureus* ATCC 12600 and *S. epidermidis* ATCC 1228. The aggregation model we propose instead may to a certain extent also be applicable to other (carbon-based) nanomaterials, like graphene (flakes) and detonation nanodiamonds. It should be noted that for bio-applications of these materials, a thorough understanding of their behaviour in complex environments is difficult to derive from standard *in vitro* experiments.

This work fits consistently with the existing body of literature on (nano)diamond-bacteria interaction. Based on the experiments, we conclude that no evidence can be found for killing on contact by milled nanodiamonds, but that observations can be ascribed to a multifactorial process of aggregation.

Declaration of competing interests

There are no conflicts to declare.

CRediT authorship contribution statement

Neda Norouzi:Investigation, Writing - original draft, Writing - review & editing.**Yori Ong**:Investigation, Writing - original draft, Writing - review & editing.**Viraj G. Damle**:Investigation, Writing - review & editing.**Mohammad B. Habibi Najafi**:Supervision, Writing - review & editing.**Romana Schirhagl**:Supervision, Project administration, Writing - review & editing.

Acknowledgements

VD acknowledges a Marie Sklodowska Marie Curie fellowship (DLV-838494) from the European Commission.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.msec.2020.110930.

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